Submission by Philip Morris U.S.A.

to

The National Toxicology Program

- Appendix 2 -

Long-Term Inhalation Study on Room-Aged Sidestream Smoke and Diesel Engine Exhaust

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Introduction

Philip Morris is funding a long-term inhalation study on room-aged sidestream smoke (RASS) and diesel engine exhaust (DEE), currently underway at INBIFO (Institut für biologische Forschung, Köln, Germany), with the objective of comparing classic and mechanistic endpoints considered to be relevant in experimental carcinogenesis.

Appended to this chapter are the following manuscripts prepared and submitted or accepted for publication based on several completed pilot studies:

- Haussmann, H.-J., Anskeit, E., Becker, D., Kuhl, P., Stinn, W., Teredesai, A., Voncken, P., Walk, R.-A., Comparison of fresh and room-aged cigarette sidestream smoke in a subchronic inhalation study on rats, *Toxicol. Sci.* 41: 100-116 (1998). **[Tab A]**

- Haussmann, H.-J., Anskeit, E., Gerstenberg, B., Göcke, W., Kuhl, P., Schepers, G., Stabbert, R., Stinn, W., Teredesai, A., Terpstra, P., Tewes, F., Twelve-month inhalation study on room-aged sidestream smoke in rats (Submitted). **[Tab B]**

- Voncken, P., Stinn, W., Haussmann, H.-J., Anskeit, E., Influence of aging and surface contact on the composition of cigarette sidestream smoke -- Models for environmental tobacco smoke, In: Dungworth, D.L., Mauderly, J.L., Oberdörster, G. (Eds.): *Toxic and carcinogenic effects of solid particles in the respiratory tract*, Washington: ILSI Press, ILSI Monographs, pp. 637-641 (1994). **[Tab C]**

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Concept of the Long-Term Inhalation Study

The study will correlate mechanistic data gained from intermediate biomarker assays with tumor development, as well as compare these data for the two test materials. The study is designed to investigate the test materials at toxicologically relevant concentrations that bear a more realistic relationship to human exposure.

This study will also contribute to the discussion on the biological plausibility of the tumorigenic risk purportedly attributable to ETS and DEE exposure. Mechanistic investigations on the interaction between the test model and the test materials will be an integral part of the study in order to provide a comprehensive interpretation of effects. The integration of mechanistic endpoints in long-term bioassays and the use of these data in risk assessment has been recommended by the NTP Board of Scientific Counselors (1992) and the International Agency for Research on Cancer (IARC) Working Group (1992), as well as in the Guidelines for Carcinogen Risk Assessment recently proposed by the U.S. Environmental Protection Agency (EPA) (1996).

The study has been designed to generally comply with regulatory requirements and recommendations (OECD guideline 451, 1986; NTP, 1991). There are two major exceptions from these guidelines in the present study design. The use of only two instead of three dose levels is motivated by the comparative nature of the study design; it is not the intention to perform a classical carcinogenesis study with the determination of a no-effect level. Also, doses have not been selected

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based primarily on the concept of maximum tolerated dose (MTD), but rather are linked to certain multiples of realistic human environmental concentrations.

In the following, critical parameters of the study concept will be discussed.

Test Atmosphere Definitions

Environmental tobacco smoke (ETS) is a complex mixture mainly composed of aged sidestream smoke (SS) as well as of small amounts of exhaled mainstream smoke (MS) (First, 1985; Baker and Proctor, 1990). Since "real" ETS cannot be generated for long-term laboratory research, RASS will be used in the long-term study as a surrogate for ETS. It will be obtained by diluting and aging SS generated from the standard reference cigarette 1R4F in a controlled, noninert environment. International standards applying to the generation of MS will be adapted to produce SS. Generally, the same chemical compounds are found in SS, RASS, and ETS, but there are quantitative differences in the concentrations and phase distribution (Guerin et al., 1992; Voncken et al., 1994).

DEE is a complex combustion aerosol like SS. The composition of DEE depends on the fuel and lubricants used as well as on the engine and its mode of use. In the long-term study, standard fuel and lubricant will be used, and international standards for diesel engine operation and the dilution of DEE will be applied (EPA, 1990).

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Both ETS and DEE are reported in the environment at similar concentrations. The most significant difference in the chemical composition of the two combustion aerosols is the insoluble carbon core of DEE particles, whereas ETS particles are almost completely soluble (Zaebst et al., 1991).

Test Atmosphere Generation

RASS will be generated from the SS of the standard reference cigarette 1R4F, a lower yield filter cigarette supplied by the University of Kentucky (Tobacco and Health Research Institute, 1990). The 1R4F is in line with current consumer preference.

Dilution and aging of SS involve physicochemical changes (e.g., particle/gas phase distribution and particle size distribution), particle losses due to deposition and adsorption on surfaces, and chemical reactions (Benner et al., 1989; Eatough et al., 1989 and 1990; Baker and Proctor, 1990). In comparison to other SS constituents, nicotine has been shown to interact most readily with surfaces (Neurath et al., 1991; Voncken et al., 1994). To quantify ETS concentrations in field studies, particle and nicotine concentrations have most frequently been determined. Due to different kinetics, the ratio of particle/nicotine mass concentrations changes with time and has thus been considered as a determinant to characterize ETS and fresh SS (Eatough et al., 1990; Nelson et al., 1992; Guerin et al., 1992; Sterling et al., 1996). Average concentration ratios of 4 up to 100 were

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determined in field studies and 2 to 4 in fresh SS.[1] This ratio may be distorted at lower ETS concentrations by unaccountable portions of nonsmoke-related particles (dust).

In order to approach "real" ETS conditions as far as possible, RASS with a mean age of at least 30 min will be used in the long-term study. A mean air residence time of 30 min (corresponding to 2 air changes per hour) can be found in less ventilated rooms.

The RASS concentrations (3 and 10 μg/l) required for the long-term study may be obtained either by aging SS at the high TPM concentration and subsequent dilution to the lower concentrations or by aging SS separately at each of the RASS concentrations required. As the intention of the long-term study is to investigate a single test atmosphere at two different concentrations, the former approach is preferred.

In our previous investigations, the chemical composition of RASS was found to change with the amount and type of surface materials in the aging room (e.g., with paper or wool) (Voncken et al., 1994). In general, particles and particle-associated components decreased, while most vapor phase components remained unchanged. In order to provide reasonable aging of the SS and to optimize the reproducibility of the setup, a noninert empty room with defined characteristics will be used in the long-term study.

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1. The ratio indicative of fresh SS was also found in previous subchronic and chronic inhalation studies on rats (Adlkofer et al., 1988; von Meyerinck et al., 1989; Coggins et al., 1992; Coggins et al., 1993; Rajini and Witschi, 1994; Witschi et al., 1995).

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In most field studies as well as in all published experimental inhalation studies, the particle concentration has been used as the key analyte to determine the concentration of ETS and ETS surrogates. In order to be able to compare our analytical and biological results with published data, TPM concentration will be used as the key analyte in the long-term study. In addition, the particle concentration has been the key analyte in all DEE inhalation studies as well as the basis for DEE risk assessment.

DEE will be generated using standard fuel and lubricant and a passenger car engine frequently in use in Europe. The engine will be driven using a standardized protocol to simulate city driving behavior.

Fresh DEE will be used as the test atmosphere to keep comparability with previously published DEE long-term inhalation studies (Karagianes et al., 1979; Iwai et al., 1986; Ishinishi et al., 1986; Vallyathan et al., 1986; Mauderly et al., 1987; Brightwell et al., 1989; Heinrich et al., 1986, 1992, and 1995; Nikula et al., 1995). Fresh DEE may be less environmentally relevant but its use in long-term inhalation studies is consistent with published literature.

A comparison of DEE composition in published inhalation studies revealed some differences. The most important difference may be the proportion of elemental carbon in the particulate phase. In the most recent published long-term DEE inhalation studies, elemental carbon was reported to be 60 % (Heinrich et al., 1995) and 92 % (Nikula et al., 1995) of TPM. It is well known that the proportion of elemental carbon in DEE particulates fluctuates (Hering et al., 1990;

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Watts, 1995). Major factors contributing to this fluctuation include, apart from emission control devices, the engine type, duty cycle, fuel, and lubricant consumption. Elemental carbon generally accounts for about 40 to 60 % of DEE particulate matter mass (Klingenberg et al., 1991; Zaebst et al., 1991).

As for SS, DEE concentrations in experimental studies are commonly based on particulate matter mass as collected on glass fiber filters. Sometimes, soot seems to be synonymously used.

Dose Levels

According to the principle of maximum tolerated dose (MTD), the highest test substance dose in a carcinogenicity study should be "sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span due to effects other than tumors. Signs of toxicity are those that may be indicated by alterations in certain serum enzyme levels or slight depression of body weight gain (less than 10 %)" (OECD Guideline 451, 1986). Setting the upper limit of doses to the MTD level should prevent toxicity from substantially interfering with tumorigenicity. On the other hand, dosing as high as this limit should prevent false negative carcinogenicity studies.

Historically, the MTD has been frequently determined in pilot subchronic/90-day studies, and histopathological lesions other than those that may be related to carcinogenesis were

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also considered (Morrow et al., 1996). However, in only approximately 20 % of the NTP inhalation studies, reduced body weight was used as the rationale for selecting the highest dose. In one study, a multiple of the human therapeutic dose was applied. In the long-term studies completed by the NTP, only 60 % had reduced body weight at the highest dose, and the magnitude of these body weight effects did not correlate with the carcinogenic response of the test substances. This is in line with recent trends within the NTP in the interpretation of the MTD concept, i.e., from the "maximum tolerated dose" to the "minimally toxic dose," which would also consider slight body weight effects in the long-term study as minimally toxic. A special workshop dealt with establishing a rationale for aerosol exposure concentrations in long-term inhalation studies (Lewis et al., 1989). Emphasis was given to substances which are relatively insoluble and of low systemic and respiratory tract toxicity. This workshop concluded that the highest dose should only minimally affect pulmonary clearance. This is in line with the concept of a functionally defined MTD (MFTD) for highest exposure concentrations in long-term bioassays (Muhle et al., 1990a), which should permit the extrapolation of observed biological effects to realistic concentrations to which humans are exposed. The MFTD concept considers biokinetic and mechanistic aspects (NTP Board of Scientific Counselors, 1992). However, more specific recommendations with regard to the type or degree of respiratory tract responses that constitute evidence of an appropriate minimally toxic response or an MFTD were not made (Lewis et al., 1989; Morrow et al., 1996). Especially, the relevance of particle overload in the lungs and the resulting inflammatory response and cell proliferation with regard to risk assessment remains open (Oberdörster, 1995).

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Altered serum enzyme levels or hematological changes as signs of toxicity were not found in a subchronic SS inhalation study at a TPM concentration of 4 μg/l (Adlkofer et al., 1988). At concentrations of up to 10 μg/l, no clinical signs of toxicity were found using "aged and diluted SS" (Coggins et al., 1993).

The rationale for the selection of the SS concentration of 4 μg TPM/l in one fully published long-term SS inhalation study was not made quite clear (Witschi et al., 1995). At this concentration, there was no significant effect on body weight. In a previous (pilot?) study performed by the same laboratory, cell proliferation was observed in bronchi and bronchioli at 1 μg TPM/l (Rajini and Witschi, 1994). The same effects as well as cell proliferation in the nasal epithelia were seen during the initial part of the chronic study (4 μg TPM/1). Obviously, the dose for the chronic study was selected based on the response in the pilot study of biomarkers considered to be related to carcinogenesis. Taking this approach for the dose selection, i.e., subchronic responses in biomarkers related to carcinogenesis, a RASS dose of 10 μg TPM/l for the present study would seem to be justified based on the biomarker responses noted above. In a second study, Witschi et al. (1997) used a TPM concentration of roughly 90 μg/l.

In the long-term study on female rats performed by Heinrich et al. (1995), the body weight of the high dose DEE group started to deviate significantly from the control on study day 200 (7 μg TPM/1, 18 hours/day, 5 days/week, weekly particle dose: 630 hours x μg/l). The final body weight difference was 17 %. Nikula et al. (1995) found a body weight gain reduction of approximately 10 % for both sexes following about 180 days of inhalation (6.5 μg TPM/1, 16

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hours/day, 5 days/week weekly particle dose: 520 hours x μg/l). This body weight gain reduction did not further increase for the male rats but increased to approximately 20 % for the female rats following approximately 500 days of inhalation. Thus, these long-term DEE inhalation studies which were both positive for lung tumors do not meet the historical MTD definition (10 % body weight gain reduction within 90 days), but are in line with the minimally toxic dose concept (body weight differences at chronic time points).

The survival of DEE-exposed rats in the study by Heinrich et al. (1995) was not affected, as was the case for female rats in the study by Nikula et al. (1995). However, the male rats in the latter study showed reduced survival in the high dose group.

Slight changes in serum clinical chemical and hematological data were reported following DEE inhalation (Ishinishi et al., 1986; Lewis et al., 1986). The relevance of these changes is unclear. These parameters have not been regularly investigated in long-term DEE inhalation studies.

DEE inhalation at the high doses mentioned above reproducibly resulted in an impairment of the macrophage-associated particle clearance from the lungs, which is connected to a particle overload phenomenon (Proceedings of Symposium on Particle Lung Interactions: Overload Related Phenomena, 1990; HEI, 1995). According to a current hypothesis, the overloaded macrophages attract PMNL to the alveolar lumen as an inflammatory response. This persisting inflammation may ultimately result in hyperplastic and metaplastic changes leading to lung fibrosis

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and carcinogenesis. The definition of an MTD or MFTD as well as the relevance of tumors arising under particle or other overload conditions for human risk assessment is under discussion. Excessive particle overload as found in high dose long-term DEE inhalation studies is certainly no condition which can be readily extrapolated to the human situation. On the other hand, prolonged particle clearance as well as associated inflammation, cell proliferation, and fibrotic changes can also be found in humans, such as coal workers, occupationally exposed to particles Oberdörster, 1995).

Deposition of soot particles in the lungs, carbon-loaded alveolar macrophages, and influx of PMNL and lymphocytes were also observed in our subchronic DEE pilot inhalation study. The magnitude of these biomarker responses suggests that the dosing regimen selected would be sufficient to elicit lung tumors in a long-term study.

The tumorigenic response to DEE in long-term inhalation studies on rats varies from 4 (spontaneous tumor prevalence) to 54 % lung tumor prevalence at approximately 7 μg particles/l (Karagianes et al., 1979; Brightwell et al., 1986; Mauderly et al., 1986; Heinrich et al., 1992). This large variance has been mainly attributed to differences in the exposure regimen (Heinrich et al. 1986), but major differences in test atmosphere generation are also probable. For weekly particle doses, a threshold of approximately 120 hours x μg/l has been suggested for lung tumorigenicity (Nikula et al., 1995). The lung soot burden is generally considered to be the major determining factor for particle-associated tumor responses. No relevant difference in the lung particle burden or associated effects were found when changing the daily exposure pattern or rate of delivery (Henderson et al., 1992). Thus, the relatively short daily exposure duration applicable to head-only

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exposure can be compensated by increasing the DEE particle concentrations over those usually used in whole-body exposure studies for longer daily durations.

Taking together the foregoing discussion on the applicability of the MTD concept to the present test atmospheres, the dose levels chosen seem to be compatible with the minimally toxic dose concept. Another aspect for setting dose levels is the intention to correlate the responses of selected biomarkers to the lung tumor response. This requires the use of doses which have been shown to be positive in previously published long-term inhalation studies. This rationale fully applies to DEE. It cannot be applied to RASS, since only few data are available on the long-term responsiveness of mice (Witschi et al., 1995; Witschi and Pinkerton, 1996; Witschi et al., 1997) but none on rats.

Maximum mean ETS concentrations in terms of smoke-related, respirable suspended particles (RSP) are reported to be approximately 0.1 μg/l in residences, offices, transportation vehicles, or other places where smoking occurs (EPA, 1992; Guerin et al., 1992). 600-1000 $\mu g/m^3$ seems to be the upper limit for the most extreme ETS concentrations reported for all types of occupied spaces. In the present study, the highest RASS particle concentration of 10 μg/l will exceed extreme human exposure concentrations by a factor of 10 and typical concentrations by a factor higher than 100. For the low dose group of this study, a TPM concentration of 3 μg/l will be used. As for the planned high RASS concentration, the high DEE concentration in the present study will exceed extreme and normal mean environmental DEE concentrations by factors of 10 and 100,

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respectively (Woskie et al., 1988; Lehmann, 1991; Pott, 1991; Elbers and Muratyan, 1991; Kühn and Bireft, 1992; projected ambient concentrations by McClellan, 1986; Brightwell et al., 1989).

Due to the comparative nature of the present study design as well as the relationship of the doses to reported environmental concentrations, the use of the same particle concentrations for both test atmospheres is considered straightforward.

Animal Model

Rats, mice, and hamsters have been preferred in carcinogenicity studies "because of their relatively short life span, . . . their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of sufficiently characterized strains" (OECD Guideline 451, 1986). The same recommendation was made by Lewis et al. (1989), particularly for aerosol inhalation studies.

For head-only exposure, rats are technically more suitable than mice and hamsters. This fact and our long experience with the rat in inhalation studies recommend its use in this long-term inhalation study. The mouse could be considered as a second species for possible future work.

In order to facilitate the detection of a low prevalence of induced tumors in the test groups, the spontaneous tumor prevalence in target organs should be as low as possible. The spontaneous prevalence of nasal cavity tumors seems to be negligible (i.e., <1 %) in the three rat

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strains considered. However, approximately 4-fold spontaneous lung tumor prevalences were reported for the Fischer 344 rat (Goodman et al., 1978; Solleveld et al., 1984; Haseman et al., 1985) compared to the Sprague Dawley (MacKenzie and Garner, 1973; Prejean et al., 1973) and Wistar rat (Vandenberghe, 1990; Kroes et al., 1988; Deerberg et al., 1980, 1982; Rehm et al., 1984; Ueberberg and Luetzen, 1979; Takizawa and Miyamoto, 1976; Boorman and Hollander, 1973; Bomhard et al., 1986). Fischer 344 rats have been used in carcinogenicity studies performed by the NTP, a preference which was based on the availability of historical data rather than scientific reasons (Gregory, 1992).

Test animals should be exposed to the test material for a major portion of their life span (OECD Guideline 451, 1986). Survival in all groups should be not less than 50 % at 24 months for rats in order for a negative test result to be accepted. In past years, the longevity of laboratory rat strains has decreased substantially, possibly due to breeding targets for rapid growth (White, 1992). The Sprague Dawley rat, which has been used in our previous MS and SS inhalation studies, is no longer considered to fulfill the above requirement (British Society of Toxicological Pathologists, 1992; White, 1992; Mariani et al., 1992). In addition, the increasingly high body weight and associated large size of adult Sprague Dawley rats do not recommend their use in a long-term head-only inhalation study.

Based on the reported low spontaneous lung tumor prevalence, the sufficient longevity and the body weight development suitable for head-only exposure, the Wistar rat seems to be particularly appropriate for the proposed long-term inhalation study. The suitability of the

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Wistar rat was corroborated in a recent historical control study. In addition, besides the Fischer 344 rat, the female Wistar rat was used in previous DEE inhalation studies (Heinrich et al., 1986, 1995).

Exposure Regimen

The head-only or nose-only exposure modes are most appropriate for rat inhalation studies with aerosols (Pauluhn, 1984; Phalen et al., 1984; Hahn, 1993). These modes diminish test substance deposition on the fur of the animals and subsequent dermal or oral uptake by grooming, which was observed following whole-body exposure (Wolff et al., 1982; Iwasaki et al., 1988; Mauderly et al. 1989; Chen et al., 1995). In a mainstream cigarette smoke inhalation study, the two exposure modes were compared: plasma and urinary nicotine concentrations were 5- to 6-fold higher in whole-body compared to nose-only exposed rats when normalized to the nicotine concentrations in the test atmospheres (Mauderly et al., 1989). In whole-body exposure, the filtering of the test atmosphere by the fur may impair reproducible uptake by inhalation. In addition, the test atmosphere is in contact with the animal excretion products. In previous studies we found that nicotine and reactive test atmosphere components such as formaldehyde are efficiently trapped by urine and feces.

Thus, to enhance the controllability of the test atmosphere administration to the rat as well as the route of the test substance uptake by the rats, the head-only exposure mode will be used in the present study.

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In inhalation studies, "long-term exposures are usually patterned on projected industrial experience, giving the animals a daily exposure of 6 hours . . . for 5 days a week (intermittent exposure)" (OECD Guideline 451, 1986). In all published DEE inhalation studies with rats, whole-body exposure was used. The daily exposure duration lasted up to 19 hours/day (Heinrich et al., 1986). Head-only exposure should not last longer than 7 hours/day due to the restraint of the rats and other technical reasons. Recently published inhalation studies on man-made vitreous fibers used head-only exposure for 6 hours/day, 5 days/week, 24 months (Smith et al., 1987; Hesterberg et al., 1993). In order to maximize our weekly doses, the present study will be conducted for 6 hours/day and 7 days/week.

Depending on the longevity of the rat strain used, it is recommended to terminate carcinogenicity studies after 24 or 30 months of exposure (OECD Guideline 451, 1986). Studies with DEE-exposed rats demonstrated that nearly 80 % of the exposure-related tumors were observed later than 24 months of inhalation (Mauderly et al., 1987). However, for the expression of tumors in this late stage of the rats' lifespan, a continuation of the inhalation period over 24 months does not seem to be necessary, since the particle load will not be substantially cleared under these conditions due to a complete loss of clearance. No information is available for SS-induced pulmonary tumorigenicity in rats, but Witschi et al. (1997) followed a similar exposure regimen for their A/J mice, i.e., inhalation followed by a postinhalation observation period. For the present study, an inhalation period of 24 months followed by a postinhalation period of 6 months is planned.

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Size of Experimental Groups

Fifty animals per sex and exposure group are considered to be sufficient to adequately evaluate a long-term bioassay (OECD Guideline 451, 1986). Assuming a spontaneous tumor prevalence of 2 %, the 51 rats per group and sex planned for the present study would be sufficient to detect an increased tumor probability of 16 % (lst order error $\alpha = 0.05$, 2nd order error $\beta = 0.10$). This is in the range of the expected lung tumor prevalence in the high dose DEE group based on the comparison of our planned weekly high DEE particle dose with published results (Karagianes et al., 1979; Iwai et al., 1986; Ishinishi et al., 1986; Vallyathan et al., 1986; Mauderly et al., 1987; Brightwell et al., 1989; Heinrich et al., 1986, 1992, and 1995; Nikula et al., 1995). Combining the results from both sexes would even allow detection of an increased tumor probability of 10 %.

Mechanistic Endpoints

In evaluating the carcinogenic risk of test materials to humans or establishing relative potencies of toxicity and carcinogenicity between species or test materials, increasing emphasis is being placed on mechanistic investigations (NTP Board of Scientific Counselors, 1992; IARC Working Group, 1992; EPA, 1996). Of special interest are preneoplastic changes that might be present before tumor manifestation or at concentrations lower than those which would be required for tumor development. These mechanistic investigations may comprise studies on pharmacokinetics, including target organ dose monitoring; genotoxicity including mutations and repair mechanisms; cell proliferation; cell differentiation; immunosuppression; and inflammatory

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or fibrotic processes. Especially for particle inhalation studies, deposition and/or clearance studies are recommended (Lewis et al., 1989).

In the present study, in addition to classical pathology, several endpoints are planned to be investigated which are thought to be mechanistically related to chronic disease and chemical carcinogenesis in the respiratory tract. The reasons for choosing these endpoints is the current mechanistic understanding of the processes under investigation as well as in-house scientific expertise. The extent of these investigations is limited by the available number of rats in a long-term head-only inhalation study.

Biomonitoring for both RASS and DEE will include the determination of the carboxyhemoglobin proportion in the blood and of the steady-state content of aminobiphenyl adducts to hemoglobin. For RASS, nicotine metabolites in urine will serve as a specific monitor. If possible, TSNA-derived hemoglobin adducts will also be investigated. In addition, the green autofluorescence of alveolar macrophages is considered for use as an estimate for the steady-state lung particle dose. The feasibility of this parameter has not yet been fully validated. .

The determination of the lung burden of inhaled particles or the pulmonary clearance efficacy are mandatory in chronic inhalation studies using particle-containing aerosols (Lewis et al., 1989). For DEE lung burden, nonlinear time-response relationships but linear dose-response relationships were found (McClellan, 1986; Heinrich et al., 1992). The nonlinearity has been

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attributed to particle overload which impairs pulmonary clearance. For SS-exposed rats, particle overload has not been published.

The oxidative modification of deoxyguanosine by forming 8-OHdG is a rather frequent event due to the permanent cellular oxidative stress (Fraga et al., 1990). Therefore, efficient repair mechanisms are set in operation which, in conjunction with the impaired catabolism of the modified base, result in the urinary excretion of 8-OH-dG. Guanidine oxidation reportedly leads to base mispairing, resulting in G:C to T:A transversions (Cheng et al., 1992). In some experimental systems, a correlation between the presence of 8-OH-dG in DNA and tumor development was observed (Floyd, 1990). This endpoint has never been investigated in long-term DEE inhalation studies to date. In the present study, both the excretion of 8-OH-dG in urine during inhalation as well as the respiratory tract tissue level of this base modification will be investigated.

Bulky DNA adducts were observed in a subchronic SS inhalation study at 10 μg TPM/l in lungs, heart, and larynx tissue using the ^{32}P-postlabeling technique (Lee et al., 1993; Brown et al., 1995). This effect was not observed at lower concentrations. This type of adduct could not be found in ETS-exposed nonsmokers (Scherer et al., 1993) but has been described for smokers (Phillips et al., 1990). DNA adducts were also detected in rat lungs following subchronic DEE inhalation (Bond et al., 1990). Although the ^{32}P-postlabeling technique seems to be the most sensitive method to detect DNA adducts, it lacks specificity. In the present study, DNA adducts will be evaluated. For this purpose, we prefer to use specific mass spectrometric methods which still

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have to be established in our laboratory. If the latter is not possible, samples will be extramurally analyzed by the ^{32}P-postlabeling technique.

Classical histopathology remains a basic endpoint for use in the present study. Apart from the microscopic confirmation and classification of tumors, persistent hyperplastic, metaplastic, and dysplastic tissue changes are considered as essential indicators of preneoplastic and neoplastic lesion induction. In subchronic inhalation studies on SS, hyperplasia and metaplasia of nasal and laryngeal epithelia were found in the rat (von Meyerinck et al., 1989; Coggins et al., 1993; Teredesai and Prühs, 1994). Due to the general reversibility of these findings after cessation of the SS inhalation, these changes have been considered an adaptive response to the irritating activity of the test atmosphere (Burger et al., 1989).

Mutations are a prerequisite for initiating carcinogenesis, and most probably also play a role in epigenetically induced carcinogenesis such as by particle overload (Driscoll et al., 1996). Apart from the latter study, there is limited expertise in the detection of early mutations in rat long-term studies. The single-strand DNA conformation assay can be used to detect unknown mutations. Recently, we have been able to increase the sensitivity of this assay by several orders of magnitude. The attempt to further increase the assay sensitivity was limited by the fidelity of the DNA polymerase used in the polymerase chain reaction to amplify DNA probes. The present assay sensitivity is not considered to be sufficient enough to detect early mutations to fulfill the function of an intermediate biomarker. However, the assay will contribute to tumor differentiation in the final part of this study. Emphasis will be given to mutations in the *p*53 tumor suppressor gene. Mutations

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of this gene can be found in about half of all human cancer cases. The location and characteristics of these mutations may reveal clues about their etiology. The predominant base changes in *p*53 in human lung cancers (G:C to T:A transversions) were suggested to be indicative of causal lesions on the nontranscribed DNA strand by polycyclic aromatic hydrocarbons (Harris, 1993). Possible correlations of *p*53 mutations with cigarette smoking have recently been discussed (Suzuki et al., 1992; Spruck et al., 1993; Habuchi et al., 1993; Brennan et al., 1995). The mutational activation of the protooncogene *ras*, another endpoint frequently associated with lung tumor development (Carbone and Minna, 1992), was not considered for the present study since the rat seems to be less sensitive to *ras* mutations by agents positive in the mouse and hamster such as N-nitrosamines (Belinsky et al., 1990).

Activation of cell proliferation, in conjunction with changes at the DNA level, is considered to be essential for initiation and tumor development. Cell division is necessary for conversion of adducts or DNA strand breaks to mutations or gaps, and also allows for mitotic recombination. However, the direct correlation between increased cell proliferation and development of neoplasia in target organs or morphological sites has been questioned (Yoshida et al., 1993). Cell proliferation, which does not give rise to formation of neoplasia, may simply be induced by the cytotoxicity of the test material in the absence of initiation. On the other hand, persistent cell proliferation may increase the probability of converting spontaneous DNA lesions to neoplastic changes. In subchronic inhalation studies on aged and diluted SS, an increased incorporation of BrdU into the rat nasal respiratory epithelium was found at a concentration of 10 μg TPM/l (Brown et al., 1995). Following a shorter period (5 days) of inhalation, the effect was

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already seen at 1 μg/l. A similar pattern of response was found for the A/J mouse at concentrations of 1 and 4 μg TPM/l (Rajini and Witschi, 1994; Witschi et al., 1995), whereas the C57BI/6 mouse did not respond. Following DEE inhalation, a transient initial increase in rat lungs was described by Wright (1986). Following chronic DEE inhalation, increased cell proliferation measured by ^{3}H-thymidine incorporation was detected in the bronchi and bronchioli as well as at particular sites in rat lungs (McClellan et al., 1986). Cell proliferation will be investigated in the present study using BrdU incorporation.

Cytokeratins have been used for the differential characterization of preneoplastic and neoplastic changes in epithelial tissues (Broers et al., 1988; Moll et al., 1988; Lindberg and Rheinwald, 1989; Schaafsma et al., 1990; Smedts et al., 1990). In contrast to the well-defined human cytokeratin expression patterns, those of the rat have been less extensively characterized. Alterations in cytokeratin expression were found to precede the histological expression of squamous metaplasia in several epithelial tissues in vitamin A-deficient rats (Gijbels et al., 1992). Recently, rat lung tumor types could be differentiated using monoclonal antibodies to human cytokeratins (Kal et al., 1993).

Inflammatory and fibrotic processes have often been associated with particle overload and carcinogenesis (Heinrich et al., 1986; Henderson et al., 1988; Muhle et al., 1990b; Morrow, 1992; Oberdörster, 1995). The PMNL proportion of lavagable bronchoalveolar cells as a sign of persistent acute inflammation was found to increase time- and dose-dependently following chronic exposure to DEE or toner.

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Lactate dehydrogenase (LDH) activity is a cellular enzyme which is commonly determined extracellularly, e.g., in the supernatant of lung lavage, as a measure for cell lysis or cytotoxicity. Increased LDH activity was found in the lavage of DEE-exposed rats (Henderson et al., 1988) and may either be ascribed to alveolar macrophage lysis or to damage to the alveolar epithelium subsequent to particle overload. The determination of lung lavage LDH activity will enhance the interpretation of data in the present study.

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